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13. ABSTRACT (Maximum 200 Words)

Enhanced heparanase expression correlates with metastatic potential, tumor vascularity and reduced postoperative survival of cancer patients. These observations, the anti-cancerous effect of heparanase gene silencing (ribozyme, siRNA) and of heparanase-inhibiting oligosaccharides, peptides and antibodies, as well as the unexpected identification of a single functional heparanase, suggest that the enzyme is a promising target for anti-cancer drug development. Our studies focused on the regulation of heparanase gene expression (i.e., promoter methylation, action of sex steroids, p53) and effect of augmented levels the enzyme on malignant behavior of prostate cancer cells. We designed effective inhibitory strategies, based on recently created chemical and molecular tools (chemically modified heparin species, siRNA-expressing vector), as well as on better understanding of biochemical aspects of heparanase proenzyme activations (inhibitory peptide approach), toward future development of effective anti-cancer therapeutic modalities.

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INTRODUCTION.

HS proteoglycans (HSPGs) and HS-degrading enzymes have long been implicated in a number of cancer- and angiogenesis-related cellular events, including cell invasion, migration, adhesion, differentiation and proliferation (1, 2). It appears that apart of direct involvement in basement membrane (BM) invasion by tumor and endothelial cells, heparanase elicits an indirect growth-promoting response by releasing HS-bound growth factors (i.e., bFGF, VEGF) from the extracellular matrix and BM, and by generating HS fragments which can potentiate bFGF receptor binding, dimerization and signaling (2). A significant correlation was found between heparanase mRNA expression and tumor vascularity in cancer patients (3). Expression of heparanase correlates with the metastatic potential of human tumor cells (1, 4, 5). Moreover, elevated levels of heparanase were detected in the urine of patients with aggressive metastatic disease. Evidence for a direct role of heparanase in tumor metastasis was provided by the increased lung and liver colonization of cancer cells following transfection and overexpression of the heparanase gene (6). Non-anticoagulant and chemically modified species of heparin and laminaran sulfate which inhibited tumor cell heparanase, also inhibited experimental metastasis (7). These observations, as well as preferential expression of heparanase in human prostate carcinoma (Fig. 1), suggest that the enzyme is causally involved in prostate cancer progression and might be a promising target for anti-cancer drug development. Our studies, described in this report, are aimed to elucidate the significance of heparanase in prostate tumorigenesis and to develop heparanase-targeting inhibitory strategies that will suppress primary growth, angiogenesis and metastasis of prostate cancer.

BODY.

1. Heparanase over-expression and prostate cancer progression.

a. Preferential expression of heparanase in primary human prostate tumors and their lymphnode and bone metastases has been demonstrated in our previous experiments (as outlined in the original application). In continuation of this study, heparanase expression was investigated in 35 cases of prostate cancer and correlated with clinical data on progression of the disease. Intense staining of heparanase was found in 21% of patients with biochemical failure (i.e., rise in PSA levels) detected 3-5 years after resection of the primary tumor, as compared to 6% of patients that showed no evidence of disease (Fig.1). These results indicate that heparanase may be applied as a prognostic marker of prostate cancer progression.

b. Transgenic mice over-expressing heparanase (8) were cross-mated with TRAMP mice. Mice positive for both genes were identified by PCR using primers specific for TRAMP and heparanase. Only 5 double homozygous male mice were identified and all developed prostate tumors and dyed within 3-4 months after birth. TRAMP mice developed tumors at a much slower rate and survived for 8-12 months (Fig. 2). The corresponding female mice were not affected. It appears that the heparanase-rich tumor microenvironment of the *hpa*-transgenic mice supports tumor take, growth rate and metastasis, but the number of mice was too small and survival time too short for establishment of a double homozygous transgenic mice colony. Metastases formation was not evaluated, since only few mice were available and spared for establishment of a colony. Unfortunately, these mice dyed early and we were unable to obtain offspring.

c. PC3 cells were stably transfected with empty pCDNA3 vector (PC3-Vo), vector encoding heparanase (PC3-hpa), or secreted form of heparanase (PC3-Sp), and injected into the right tibia of the male SCID mice. Tumor growth was monitored for 5 weeks. PC3-Sp cells and, to the less extent, PC3-hpa cells formed well-established osteolytic tumors, while small or no tumors were detected in mice injected with control PC3-Vo cells (Fig. 3A).

In additional experiment, CWR prostate carcinoma cells were stable transfected with control vector (CWR-Vo), or vector encoding the heparanase cDNA (CWR-*hpa*). Six-week-old male Balb/nude mice received an intracardiac injection of CWR-Vo, or CWR-*hpa* cells and survival rate of the mice was monitored for 18 weeks following cell injection. Mice injected with CWR-*hpa* cells developed secondary tumors in distant sites vs. no detectable tumors in CWR-Vo injected mice, as also reflected by the marked difference in survival time (Fig. 3B). Thus, over-expression of heparanase in prostate cancer cells is associated with a marked increase in malignant potential and mortality. However, bone metastasis per se could not be detected following intracardial inoculation of heparanase over-expressing cells (CWR, PC3). Presently, we are developing cell models of prostate cancer genetically engineered to express luciferase reporter gene, so that *in vivo* whole-body bioluminescent reporter imaging can detect bone metastases, without having to use histology. These models will help us to elucidate a role of heparanase in promoting prostate carcinoma metastasis.

2. Regulation of heparanase promoter activity in prostate cancer.

i. Testosterone induces heparanase promoter activity in prostate cancer cells. We have identified 2 putative androgen response elements (ARE) in the 5' flanking region of the heparanase gene, located at positions –1540 and –1370 relative to the transcription initiation site (Fig. 4, *top*). To investigate the effect of androgens on *hpa*-transcription, we constructed a DNA vector in which the *hpa*-promoter region (1.8 kb) containing the AREs is introduced in front of a luciferase reporter gene and demonstrated that this region is sufficient to support reporter gene expression in transiently transfected CWR cells. A 4-fold increase in transcriptional activity of the *hpa*-promoter was obtained in AR positive CWR cells in response to a physiological concentration of testosterone (0.1 nM) (Fig. 4). In contrast, there was no effect in AR-negative DU145 cells. Androgen treatment also induced a 3-4-fold increase in *hpa*-mRNA expression in CWR cells, confirming the promoter study data.

ii. Heparanase promoter activity is regulated by p53 in prostate cancer. Mutational inactivation of p53 is the most frequent genetic alteration in human cancers. To investigate whether heparanase gene expression is affected by p53, CWR prostate cancer cells were co-transfected with the reporter vector described above, and a pCDNA3 plasmid encoding the wt p53. Luciferase activity was determined 24 h later, revealing a marked decrease in transcriptional activity of the *hpa*-promoter and enzymatic activity of heparanase (Fig. 5). These results indicate that heparanase gene expression in prostate cancer cells is upregulated upon inactivation of p53. In another set of experiments, we compared endogenous heparanase (hpa) expression in murine embryonic fibroblasts (MEF) derived from wt and p53 knock out ($p53^{-/-}$) mice. Heparanase mRNA expression, assessed by semiquantitative reverse transcription polymerase chain reaction (RT-PCR), was markedly elevated in $p53^{-/-}$ MEF (Fig. 6A). Moreover, heparanase enzymatic activity in cell lysates of $p53^{-/-}$ MEF was significantly higher as compared to wt MEF (Fig. 6B).
p53 may exert its inhibitory effect on heparanase gene by either direct binding to the heparanase promoter or indirectly, by binding to and inhibition of transcription factors that activate the heparanase promoter. In order to test the possibility of a direct interaction of p53 with heparanase promoter, we performed the chromatin immunoprecipitation (ChIP) analysis. Using heparanase promoter-specific PCR primers, directed to the putative p53-binding sites, we found enrichment of the promoter sequence in nuclear extracts from WI-38 cells, immunoprecipitated with antibody against p53, as compared to nuclear extracts from WI-38 cells stably transfected with a genetic suppressor element 56 (WI-38/GSE56)

(Fig. 7). It was previously reported that expression of GSE-56, which corresponds to the C-terminal portion of p53 (residues 275-368), results in the accumulation of p53 in its inactive conformation and in inhibition of p53 interaction with target promoter sequence. This set of experiments demonstrated physical association between heparanase gene regulatory sequence and p53, confirming direct regulation of heparanase expression by p53.

3. Heparanase involvement in β-catenin nuclear trafficking and the down regulation of Wnt signaling. We have demonstrated that heparanase specifically binds β-catenin and γ-catenin in the cytoplasm and nucleus of prostate cancer cells. To elucidate a possible effect of heparanase-β-catenin interaction on Wnt signaling, *hpa*-transfected CWR prostate cancer cells were transiently transfected with a pGL2 LUC-hpa promoter reporter construct containing three TCF binding sites. A pGL2 plasmid containing 3 mutated TCF binding sites was used as a control. Inhibition of Wnt signaling was reflected by a 3-6 fold decrease in transcriptional activity obtained in the *hpa*-transfected vs. control cells. Heparanase involvement in the regulation of Wnt signaling was further supported by down regulation of c-myc in the *hpa*-transfected vs. mock transfected cells.

4. Heparanase-inhibiting strategies

i. Inhibition of heparanase by glycol-split species of heparin. Inhibition of heparanase by chemically modified species of heparin with increased percentage of N-acetylation has been studied in our experiments and found to reduce the heparanase-inhibiting activity of heparin, indicating that N-sulfate groups contribute to this inhibitory activity. On the basis of structure-activity relationship emerging from these studies, our collaborators (Dr. Benito Casu, 'Ronzoni' Institute, Milan, Italy) removed excess sulfate groups, thus improving the molecular flexibility of species of heparin, and applied controlled glycol-splitting and sulfation/desulfation strategies. Best results in terms of specificity and inhibitory activity (complete inhibition of heparanase activity at 0.1 μg/ml) were obtained with compound with 100% N-desulfation/N-acetylation & 20% glycol-split. This compound, unlike other species of heparin, was selected as a lead compound also by virtue of its non-anticoagulant property and inability to promote angiogenesis through release of ECM-bound bFGF and stimulation of bFGF-receptor binding and mitogenic activity. This compound was highly effective in inhibiting experimental metastasis (an almost complete inhibition of lung

colonization at 100 μg/mouse) and wound angiogenesis, evaluated by MRI analysis of vascular density and functionality.

ii. Heparanase gene silencing averts tumor invasiveness, angiogenesis and metastatic spread. We employed gene-silencing strategies to demonstrate the contributory role of heparanase in malignancy and explored the therapeutic potential of its specific targeting. For this purpose, we designed plasmid vectors that enable effective expression of small interfering (si) RNA directed against the human and mouse (9) heparanase mRNAs. Several human and mouse tumor cell lines, including prostate, with either naturally elevated levels of the endogenous enzyme, or genetically engineered to overexpress heparanase, were transfected with anti-*hpa* siRNA. Semiquantitative RT-PCR and measurements of enzymatic activity revealed efficient silencing of *hpa* gene and protein expression, and a profound inhibition of heparanase activity in human prostate carcinoma cells. *Hpa* gene silencing led to a profound decrease in tumor cell invasion and adhesion *in vitro*, as well as in tumor vascularization and metastasis *in vivo*, resulting in a prolonged survival of the tumor bearing mice. These data represent the first successful application of siRNA-mediated heparanase gene silencing and highlight the decisive role of heparanase in cancer metastasis and angiogenesis. Heparanase gene silencing, acting on the RNA level, is of particular advantage in providing mechanistic and functional insights into non-enzymatic functions of heparanase (i.e., cell adhesion, survival signals), unobtainable by the currently available inhibitors of heparanase enzymatic activity.

iii. Peptides and point mutations inhibiting heparanase proteloytic processing and activation. Peptides corresponding to the potential cleavage region were synthesized and tested for their ability to competitively inhibit the cleavage reaction. A synthetic peptide (LREHYQKKFKN) was found to inhibit processing and activation of the 65 kDa latent enzyme. An adjacent peptide (FDPKKESTFEERFDER) had no inhibitory effect. Proteolytic cleavage of the 65 kDa proenzyme occurs in two potential cleavage sites, Glu^{109}-Ser^{110} and Gln^{157}-Lys^{159}. In order to elucidate whether heparanase processing and activation involve two distinct sites and identify amino acids that are essential for heparanase processing, we introduced point mutations in each of the two potential cleavage sites and their adjacent amino acids. Heparanase negative cells (JAR human choriocarcinoma) were transfected with each of the point mutated full-length constructs and tested for heparanase processing (Western blotting) and enzymatic activity. Our results indicate that point mutations in site 2 and its vicinity affect heparanase processing as reflected by lack of processing (i.e., Q^{157}---K; Y^{156}---A) and lack of enzymatic activity in the *hpa*-transfected JAR cells. On the other hand,

various point mutations and deletions in site 1 had no effect on heparanase processing and activation.

KEY RESEARCH ACCOMPLISHMENTS.

i) Heparanase has been characterized as a promising prognostic marker of prostate cancer progression.

ii) Causal involvement of heparanase in prostate tumorigenesis has been demonstrated in various *in vivo* systems (spontaneous tumor formation in transgenic mice, transplantable tumor models).

iii) We found that heparanase expression in prostate is tightly regulated at the level of promoter activity. In particular, testosterone and p53 have been identified as critical determinants of heparanase promoter induction/inhibition (in accordance).

iv) Several anti-heparanase inhibitory strategies were developed and tested:

- enzymatic inhibition approach: chemically modified species of heparin with increased heparanase-inhibiting ability but lacking other biological activities of normal heparin (i.e., anticoagulant, pro-angiogenic) that are irrelevant or undesirable in the case of anti-tumor drug development.
- gene-silencing approach: short inhibitory RNA (siRNA) characterized by increased stability and specificity of targeting.
- inhibition of proteolytic processing of pro-heparanase enzyme, based on precise characterization of its cleavage site(s).

REPORTABLE OUTCOMES.

Abstract: chosen for presentation in Minisymposium session at the 2005 AACR Annual Meeting (Anaheim, California).

Funding applied for, based on work supported by this award: Israel Cancer Research Fund postdoctoral fellowship (Lea Baraz) –Awarded (January 2005).

CONCLUSIONS.

In the course of the first year of research we addressed each of the tasks outlined in original Statement Of Work, toward a better understanding of the involvement of heparanase in prostate tumorigenesis. The experimental systems and tools established during this year provide a necessary basis for continuation of the proposed research. These include: establishment of prostate carcinoma *in vivo* models and demonstration of the contributory role of heparanase in prostate tumor progression [task 1]; establishment of experimental cellular systems to study transcriptional control of heparanase, and characterization of heparanase promoter regulation through molecular mechanisms highly relevant to prostate tumorigenesis [task 2]; development of tools to study recently emerging non-enzymatic activities of heparanase in prostate cancer progression (involvement in β-catenin nuclear trafficking and cell signaling pathways) [task 3]; design of effective inhibitory strategies, based on recently developed chemical and molecular tools (chemically modified heparin species, siRNA-expressing vector), as well as on better understanding of biochemical aspects of heparanase proenzyme activations (inhibitory peptide approach) [task 4].

"So what": the results obtained during the first year of research clearly support the involvement of heparanase in prostate tumorigenesis, as hypothesized in our original application. A conclusive indication is the accelerated prostate carcinoma development and progression in various hpa-overexpressing animal models. Growing understanding of the regulatory machinery of heparanase promoter is of high significance, as a part of general effort to define the exact molecular mechanism of heparanase-driven prostate tumorigenesis. Inhibitory strategies designed in the course of the research are aimed to further development into effective anti-cancer therapeutic modalities, based on anti-heparanase approach.

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APPENDIX

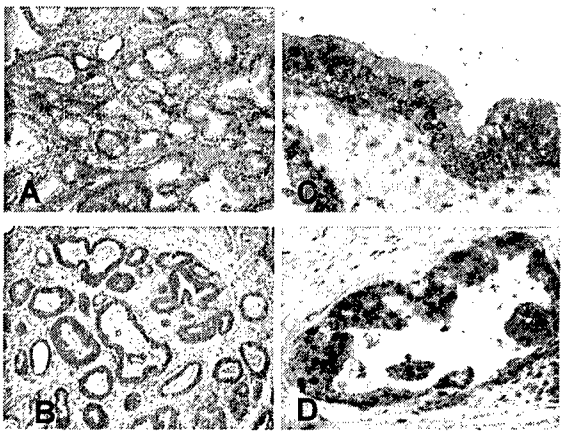


Fig. 1 Expression of heparanase in human prostate cancer. B-D. Intense staining of heparanase was found in 21% of patients with biochemical failure (i.e., rise in PSA levels) detected 3-5 years after resection of the primary tumor, as compared to 6% of patients that showed no evidence of disease. Nuclear localization of heparanase is noted in some of the cells (D).

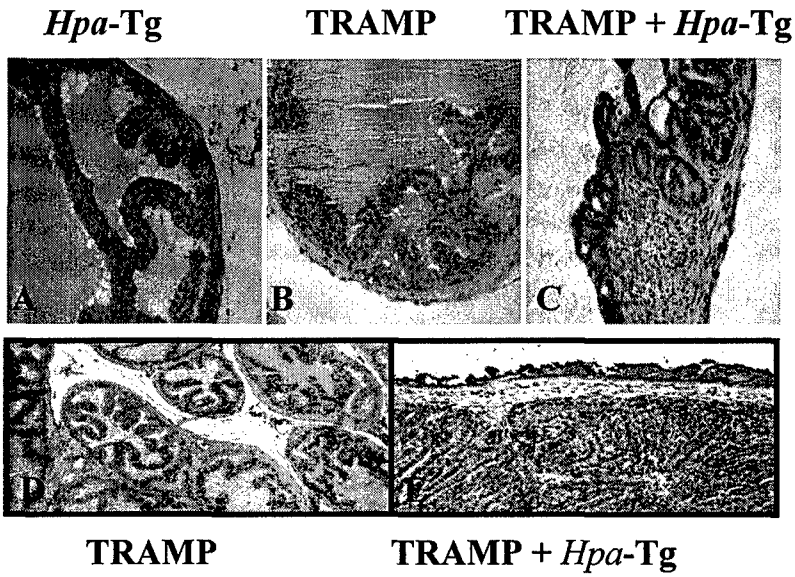


Fig. 2 Involvement of heparanase, expressed by the tumor (TRAMP) and stromal (*hpa*-tg) compartments, in prostate cancer progression. Transgenic mice over-expressing heparanase (*Hpa*-tg) were cross-mated with TRAMP mice. Double homozygous male mice were identified by PCR using primers specific for TRAMP and heparanase. Cross-mating accelerated tumor formation and induced a pronounced stromal response (C). Over-expression of heparanase (immunostaining) is noted in the prostate epithelium of the cross-mated mice (E).

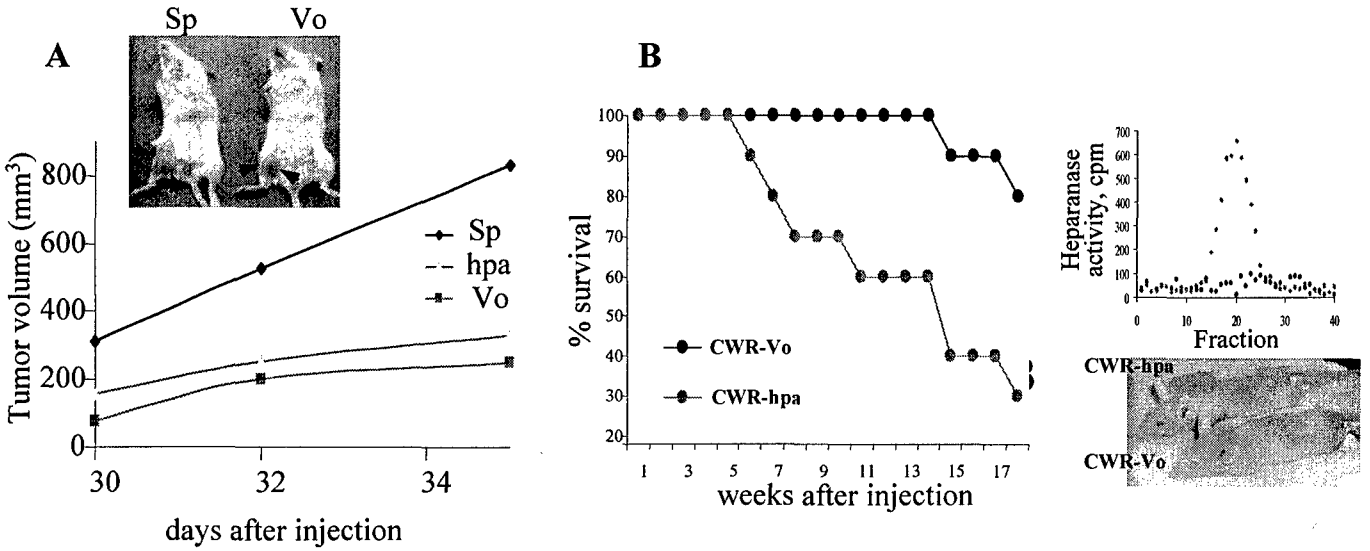


Fig. 3: Over-expression of heparanase accelerates prostate tumor formation and mortality. **A**. PC3 cells stably transfected with empty vector (PC3-Vo), vector encoding heparanase (PC3-hpa), or secreted form of heparanase (PC3-Sp) were injected into the right tibia of the SCID mice. Tumor size was monitored for 5 weeks. *Inset*: representative tumors (arrowheads). **B**. Balb/nude male mice received an intracardiac injection of CWR cells transfected with *Hpa*-pcDNA plasmid (CWR-*hpa*) or vector alone (CWR-Vo) High heparanase activity has been detected in CWR-*hpa*, but not in CWR-Vo cells (right, top). Mice injected with CWR-*hpa* cells developed secondary tumors in distant sites vs. no detectable tumors in CWR-Vo injected mice (right, bottom), reflected by a marked difference in survival time (left).

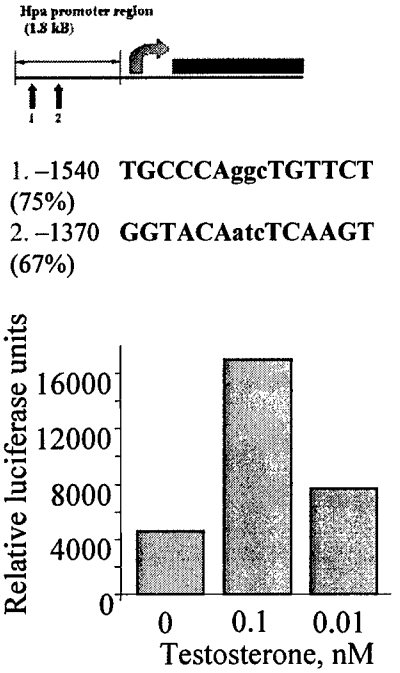


Fig. 4 Testosterone increases heparanase promoter activity. *Top:* Structure of the human heparanase gene (*Hpa*) promoter region. Red arrows (1 and 2) represent regions with >60% homology to the consensus ARE. *Bottom*: CWR cells were transiently transfected with LUC reporter gene driven by heparanase promoter (pGL2-*Hpa*). After overnight recovery, cells were treated with testosterone (1×10^{-7}; 1×10^{-8} M). LUC activity was determined 24 h later and is expressed in relative light units.

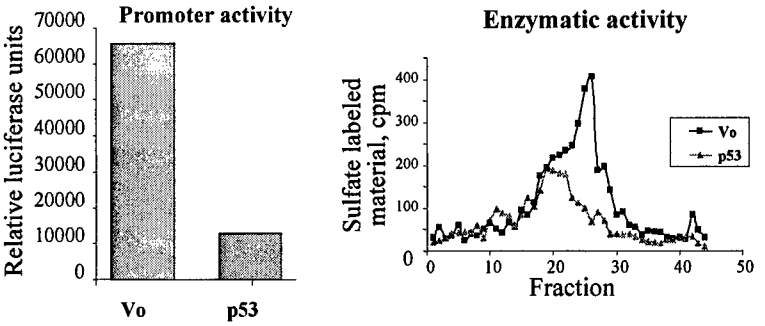


Fig.5. Overexpression of p53 represses heparanase promoter and enzymatic activity. **A.** CWR cells were co-transfected with a luciferase reporter gene driven by the heparanase promoter and with vectors encoding for p53 protein or empty vector alone, Vo. Luciferase activity was determined 24 h later. The relative light units determined in each sample was then normalized against beta-galactosidase activity measured by a colorimetric assay. **B.** Heparanase activity. p53 negative SaoS-2 cells were transfected with a control vector or vector encoding wt p53 and heparanase enzymatic activity (release of HS degradation fragments) was tested 24 h later, as described in Fig. 1A

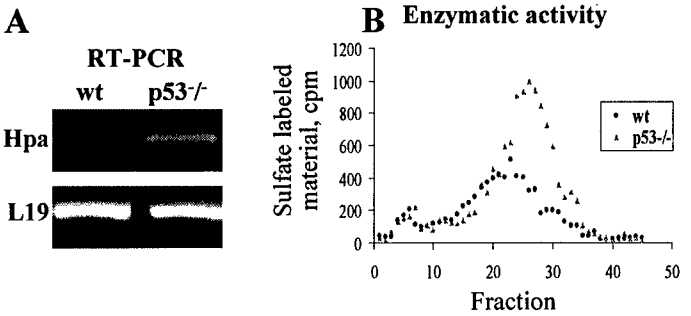
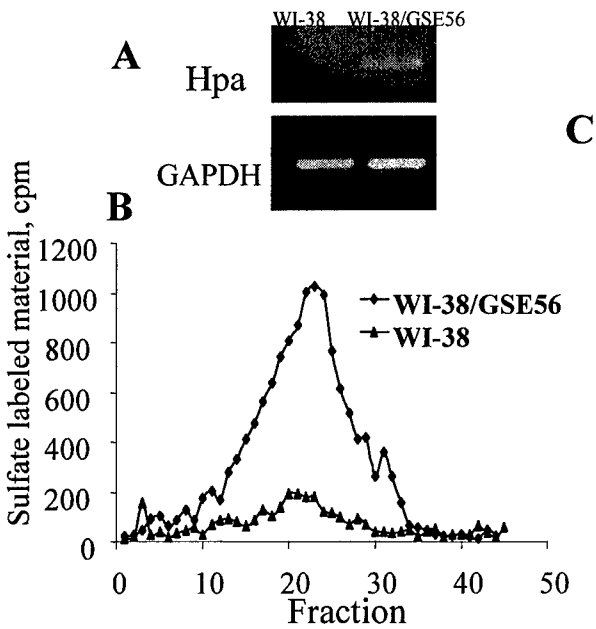


Fig. 6. Increased heparanase expression in p53 knock out murine embryonic fibroblasts (MEF). **A.** Hpa) mRNA expression. RNA isolated from wt and $p53^{-/-}$ MEF, was reverse transcribed to cDNA and subjected to comparative semiquantitative PCR. Aliquots (10 µl) of the PCR products were separated by 1.5% agarose gel electrophoresis and visualized. **B.** Enzymatic activity. MEF ($1x10^6$ cells), obtained from wt or p53 knock out ($p53^{-/-}$) mice were tested for heparanase activity, as described elsewhere.



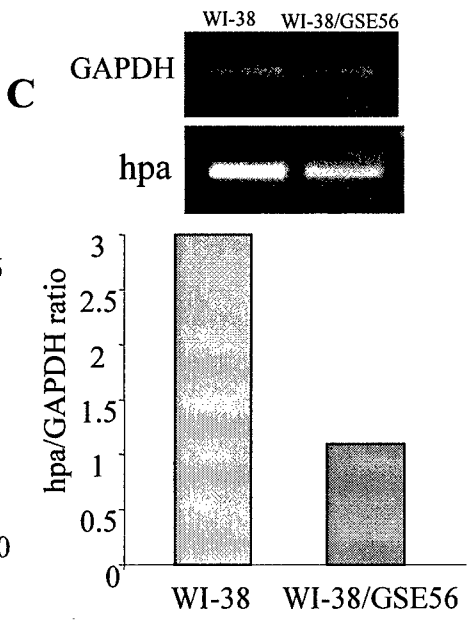


Fig. 7. WI-38 cells were stably transfected with GSE-56. Hpa mRNA expression (A), and enzymatic activity (B) in WI-38/GSE56 cells were significantly higher than in parental WI-38cells. Chromatin Immunoprecipitation (ChIP) analysis with Hpa promoter-specific PCR primers, directed to the putative p53-binding sites, revealed enrichment of the promoter sequence in nuclear extracts from WI-38 cells, immunoprecipitated with antibody against p53, as compared to nuclear extracts from WI-38/GSE56 cells.